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METHODS

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Promoters and regulatory elements that improve adeno-associated virus transgene expression in the brain

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Abstract

Since the first demonstration of central nervous system (CNS) transduction with recombinant adeno-associated virus, improvements in vector production and promoter strength have lead to dramatic increases in the number of cells transduced and the level of expression within each cell. The improvements in promoter strength have resulted from a move away from the original cytomegalovirus (CMV) promoter toward the use of hybrid CMV-based promoters and constitutive cellular promoters. This review summarizes and compares different promoters and regulatory elements that have been used with rAAV as a reference toward achieving a high level of rAAV-mediated transgene expression in the CNS.

Keywords: Adeno-associated virus; Regulatory element; Promoter; NSE; CBA; Brain; CNS; Transgene; Glia; Neuron

1. Introduction

Recombinant adeno-associated virus (rAAV) has become an attractive vehicle for delivering transgenes to the central nervous system (CNS) due to its lack of toxicity and absence of inflammatory response. Constant improvements in rAAV vector technology have allowed high-titer, high-purity vectors that are free of wild-type AAV and adenovirus to be produced. These features, combined with the ability of rAAV to stably transduce a wide variety of neuronal cell types in many brain areas, have facilitated phenotypic correction in many rodent disease models.

The level of transgene expression within the rAAV-transduced cell can be critical. In some instances the expression cassette needs to be constantly regulated to maintain a therapeutic level of protein. This obstacle has been approached by using regulated gene expression systems that can tightly modulate the level of therapeutic protein by administration or removal of a drug. Conversely, disorders such as those caused by enzyme deficiencies may require a high level of sustained expression to approach normal protein levels in the brain. The aim of this review is to provide a summary and comparison of promoters and regulatory elements that have been used with rAAV in the brain as a guide to achieving optimal gene expression.

2. Viral-based promoters

2.1. Cytomegalovirus promoter

Early rAAV-based studies in the brain utilized the cytomegalovirus immediate early promoter and enhancer (CMV promoter) which had been previously demonstrated to drive strong expression in the brain [1]. The small size of 700 bp allowed transgenes of approximately 3.4 kb to be packaged into rAAV. A summary of studies using the CMV promoter is presented in Table 1.

In the first demonstration of rAAV-mediated transduction of brain cells, Kaplitt et al. [2] injected a rAAV vector containing tyrosine hydroxylase (TH) under control of the CMV promoter into the rat striatum. This resulted in the transduction of approximately 1000 TH-positive neurons at 3 weeks postinjection, although expression was somewhat diminished after 4 months.

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Table 1
Use of viral or viral-derived promoters for rAAV-mediated transduction of the CNS

Promoter	Transgene	Virions injected	Brain area(s) targeted	Expression level and duration	Reference
CMV	TH	1×10^4 TU	Rat striatum	TH IHC revealed $>1 \times 10^3$ TH +ve cells after 1 week; expression was significantly decreased by 4 months (NQ)	[2]
CMV	*lacZ*	5×10^4 TU	Rat olfactory tubercule, inferior colliculus, hippocampus, piriform cortex and striatum	Olfactory tubercule, 10–20 *lacZ* +ve cells per 40-μm section; inferior colliculus, 20–50 cells per section; expression in both brain areas was stable over 3-month experiment. Hippocampus and piriform cortex, >20 cells per section; striatum, <10 cells per section; expression in these three brain areas decreased after 3 weeks	[3]
CMV	$GABA_A$ Rα-1 subunit	3×10^9 particles	Rat inferior colliculus	IHC with anti-$GABA_A$ receptor α-1 subunit antibody revealed extensive expression in the inferior colliculus after 7 days (NQ)	[4]
CMV	GFP	3×10^2 TU	Rat parabrachial nucleus	340 GFP +ve cells when every fourth 40-μm section was counted after 2 weeks. Expression was stable over 3-month experiment	[5]
CMV	TH-IRES-AADC	4.5×10^5 IU	Monkey striatum	IHC of FLAG-tagged AADC revealed expression in numerous striatal neurons (NQ). Expression decreased after 2 months but was still detectable after 4 months	[6]
CMV	*lacZ*	1×10^7 IU	BALB/c mouse striatum	Hundreds of *lacZ* +ve cells at 2 months. Expression decreased at 4–6 months although a few *lacZ* +ve cells still detected in some brains at 12 months (NQ)	[7]
CMV	GUSB	2×10^5 TU	MPSVII mouse striatum	GUSB activity 133% of heterozygous mice, enzyme activity spread from 2 mm at 6 weeks to 4 mm after 4 months	[8]
CMV	GUSB	4×10^8 particles	MPSVII mouse striatum/cortex, thalamus/hippocampus	GUSB activity was 8–12% of normal; expression stable over 5 months	[9]
CMV	GUSB	1.5×10^{11} particles	Delivery into CSF of neonatal MPSVII mice	GUSB activity in brain was ~10% of normal after 1 week and ~25% of normal after 4 months	[10]
CMV	GFP	2.5×10^5 TU	Rat striatum (anterior and posterior)	GFP +ve cells detected in the striatum, globus pallidus internal capsule (GPi), and stria terminalis. An average of 366 GFP +ve cells were detected in the striatum after 1 month. Expression decreased after 1 month but persisted at least 11 months. Expression in GPi was fivefold higher that in striatum	[11]
MD	GDNF	2×10^9 particles	Rat substantia nigra	~1×10^3 pg GDNF detected by GDNF ELISA which was ~10-fold higher than that in control rats and stable over 3- to 10-week experiment	[14,15]
MD	TH, GTPCHI	1×10^8 particles	Rat striatum	TH IHC revealed ~4×10^3 TH +ve cells at 3 weeks and 1×10^3 at 6 months. Expression persisted >1 year (NQ)	[16]
MD	AADC	3.6×10^9 particles	Rat striatum	AADC IHC revealed thousands of AADC +ve cells at 3, 6, and 12 months (NQ). AADC assay showed expression stable for >26 weeks	[17]
MD	GDNF	4×10^9–9×10^9 particles	Rat striatum and substantia nigra	GDNF IHC on striatally injected sections revealed widespread staining through striatum and through cortex and globus pallidus over 6-month experiment (NQ). GDNF ELISA after 4 weeks showed activity was 4- to 35-fold above baseline	[18]

CMV-β-globin intron	AADC	3.6×10^{11} particles	Monkey striatum	AADC IHC revealed 8–16 $\times 10^6$ AADC +ve cells after 8 weeks. Positron emission tomography with an AADC tracer showed that expression was stable >12 months	[19,20]
CMV-β-globin intron	AADC	4×10^9 particles	Rat striatum	AADC IHC revealed numerous AADC +ve neurons in the striatum after 3 weeks (NQ)	[21]
CMV-hGH intron	TH, AADC	3×10^9 particles	Rat striatum	TH and AADC IHC revealed numerous TH and AADC +ve neurons in the striatum after 6 weeks (NQ)	[22]
CMV-hGH intron	TH, GTP-CH1, AADC	1.5×10^8 particles	Rat striatum	TH IHC revealed 2–4 $\times 10^4$ TH +ve neurons in striatum at 7 months and a similar level after 12 months (NQ)	[23]
CBA	GFP	2.7×10^8 particles	Mouse cerebellum	Extensive GFP +ve in Purkinje cells of cerebellum after 1 week (NQ)	[28]
CBA	GFP	3×10^8 IU	Rat hippocampus	Extensive GFP +ve cells in CA1 pyramidal neurons after 7 months (NQ)	[29]
CBA	GUSB	1.5×10^{11} particles	Intravenous delivery into MPSVII mice	GUSB activity 25% of normal and stable from 1 week to >1 year	[30]
CBA	GFP, α-synuclein-A30P-IRES-GFP	3×10^{10} particles	Rat substantia nigra	Extensive GFP +ve and α-synuclein-+ve cells detected through a major fraction of the substantia nigra with expression remaining stable over 1 year experiment (NQ). GFP+ve and α-synuclein +ve nigrostriatal axons were present throughout striatum	[31]
CBA	GFP, α-synuclein	1.6×10^9 IU	Rat substantia nigra	Extensive GFP +ve cells detected through substantia nigra with expression peaking at 8 weeks and remaining stable over 27-week experiment (NQ). GFP +ve fiber terminals filled striatum. Expression similar for α-synuclein with >90% of TH +ve neurons also expressing α-synuclein	[32]
CBA	GFP	5×10^9 particles	Rat hippocampus	~2×10^5 GFP-expressing cells detected in hippocampus after 1 month with expression stable >18 months. Threefold more CBA–GFP-expressing cells detected than NSE–GFP-expressing cells with matched titers.	[33]
MFG	NGF	3.4×10^9	Rat medial septum	87% cholinergic neuron survival after fimbria fornix lesion compared to 23% survival in control rats (NQ)	[35]
RSV	*lacZ*	2×10^9–4×10^9 particles	Rat lateral ventricle and striatum	<10 *lacZ* +ve ependymal cells and <20 *lacZ* +ve striatal cells after 3 and 15 weeks	[36]
RSV	bcl-2	1.7×10^{10}–2.4×10^{11} particles	Gerbil hippocampus	Extensive FLAG-tagged bcl-2 detected by IHC in CA1 pyramidal cells after 5 days (NQ)	[37]

All times stated refer to the length of time after AAV administration. ELISA, enzyme-linked immunosorbent assay; +ve, positive; IHC, immunohistochemistry; IU, infectious units; TU, transducing units; NQ, transgene expression was not quantified; GDNF, glial-derived neurotrophic factor; GTPCHI, GTP cyclohydrolase I; IRES, internal ribosome entry site; GUSB, β-glucuronidase. All other abbreviations are referred to in the text. The titer refers to the total amount of virus injected. Viruses titered by different methods are measured in different units. Virus titered using the infectious center assay is measured in IU; virus titered by a functional assay, such as counting immuno-positive cells after infection of a cell line with rAAV, is measured in TU; virus titered by dot blot or quantitative PCR to quantify number of genomes is measured in particles; virus titered by an ELISA using an antibody to the capsid is referred to as capsid titer. For further explanation see [38].

Transduction of various distinct brain areas was demonstrated by McCown et al. [3] who found that 3 weeks after injection of rAAV–CMV–*lacZ* into the olfactory tubercule, striatum, hippocampus, piriform cortex, and inferior colliculus of mice, *lacZ* expression was detected in each brain area, ranging from less than 10 cells per 40-μm section in the striatum to over 50 cells per 40-μm in the inferior colliculus. Three months postinjection, however, expression was substantially reduced in the hippocampus and piriform cortex and somewhat decreased in the striatum, suggesting that the level and duration of rAAV-mediated expression was specific to different brain areas.

There have since been many studies using the CMV promoter to drive transgene expression. Many studies where expression was monitored for a short time have demonstrated stable CMV-driven expression in many brain regions facilitating at least partial phenotypic recovery [8–10] or protecting from insult [4]. Studies monitoring expression over the long term, however, were plagued by declining expression over time [2,3,7]. This phenomenon has been ascribed to silencing of the viral-derived promoter by methylation based on a study by Prösch et al. [12] who showed that the CMV promoter was susceptible to transcriptional inactivation by methylation of cytosines in CpG dinucleotides. The observed promoter inactivation may also have been due, at least in part, to impurities in early vector preparations. It is also important to note that lentiviral vectors containing the CMV promoter have been demonstrated to sustain long-term expression in the rat brain [13]. The recent characterization of other promoters that drive a higher level of sustained AAV-mediated expression in the CNS has precipitated the phasing out of the original CMV promoter.

2.2. CMV-derived promoters

Modifications of the CMV promoter have been engineered in an effort to both enhance and stabilize gene expression. The (MD) promoter consists of the CMV promoter fused to β-globin exons two and three and an intervening intron. This promoter has been employed by the Mandel research group to transduce nigral and striatal neurons to test various transgenes for efficacy in a rat model of Parkinson's disease. Robust transduction of thousands of neurons was achieved and, furthermore, transgene expression was stable for greater than 1 year [14–18] (Table 1).

A similar promoter that contains the CMV promoter and a chimeric intron composed of a CMV splice donor and a human globin splice acceptor was employed by Bankiewicz et al. [19] and Sanchez- Pernaute et al. [21]. Injection of a rAAV vector containing aromatic L-amino-acid decarboxylase under control of this promoter resulted in transduction of approximately 8–16 million neurons in the monkey striatum with expression persisting for at least 1 year [20].

Long-term expression was also observed when the CMV promoter was fused to the human growth hormone first intron. Approximately 20,000–40,000 striatal neurons were transduced when rAAV-TH was driven by this promoter with no attenuation of expression after 1 year [23].

Based on the similarities in results from all of these studies, it is likely that the increased expression levels and persistence of expression was due to stabilization of the CMV promoter by addition of an intron that may improve the efficiency of RNA processing [24].

2.3. CMV enhancer/chicken β-actin promoter

The CMV enhancer/chicken β-actin promoter (variously called CBA, CB, or CAG) was first described by Niwa et al. [25] as a strong constitutive promoter consisting of the chicken β-actin promoter fused downstream of the CMV enhancer. A 1.7-kb version of this promoter containing the CMV enhancer/chicken β-actin promoter fused to 90 nucleotides of exon one of the chicken β-actin gene, 917 nucleotides of a hybrid chicken β-actin/rabbit β-globin intron, and 55 nucleotides of rabbit β-globin exon three has been most commonly employed for gene therapy applications [26,27]. In the past few years, rAAV in conjunction with CBA has been shown to facilitate a high level of rAAV-mediated gene expression in various brain areas (Table 1).

In an initial study using CBA to promote rAAV-mediated gene expression in the brain, Kaemmerer et al. [28] demonstrated transduction of numerous Purkinje cells in the mouse cerebellum. By comparison, few green fluorescent protein (GFP) positive cells were observed after injection of a similar cassette driven by the CMV promoter. The authors hypothesize that this may have been due to transcriptional inactivation of the CMV promoter as coinjection with adenovirus serotype five did allow transduction of some Purkinje cells, possibly by activation of the CMV promoter by the adenovirus E1 protein.

The CBA promoter also facilitates stable, long-term gene expression. Extensive transduction of CA1 pyramidal cells in the rat hippocampus was observed 7 months after injection of rAAV [29] and therapeutic levels of β-glucuronidase (GUSB) persisted greater than one year after intravenous administration into MPSVII mice [30]. In two recent studies characterizing a new model of Parkinson's disease, AAV–CBA mediated overexpression of GFP led to transduction of the majority of the substantia nigra, filling the entire striatum with GFP positive terminals. GFP expression peaked by 1 [31] to 2 [32] months and remained at a constant level over the 6-month [32] or 1-year [31] duration of the study.

Klein et al. [33] directly compared the number of GFP-expressing cells in the rat hippocampus resulting from use of the CBA compared with the neuron-specific enolase (NSE) promoter (see Section 3) and found a 3.2-fold greater number of transduced cells when the CBA promoter was used. Our research group has qualitatively observed a 1.1-kb version of the CBA promoter with a shorter intron (derived from pBacMam3, Novagen, Madison, WI) to drive high levels of GFP in hippocampus, substantia nigra, and striatum (unpublished observations) at a level comparable with that of the NSE promoter. Furthermore, Björklund et al. [34] have found that the 1.7-kb CBA promoter in conjunction with the woodchuck post-transcriptional regulatory element (WPRE; see Section 4) provides a 7- to 50-fold higher transduction efficiency in striatum and substantia nigra than the MD promoter used in their previous studies.

2.4. Other viral promoters

In the context of rAAV, the Moloney murine leukemia virus long terminal repeat (MGF) and the Rous sarcoma virus long terminal repeat (RSV) promoters have been less well characterized. Injection of nerve growth factor under control of the MGF promoter into the medial septum has been shown to facilitate a 60% increase in survival of cholinergic neurons after fimbria–fornix lesion although the level of transgene expression was not measured directly in vivo [35].

The RSV promoter was found by Davidson et al. [36] to drive weak expression after injection into the rat lateral ventricle and striatum with only 10–20 *lacZ* positive cells detected; however, in another study the RSV promoter drove robust expression of bcl-2 in numerous CA1 pyramidal cells of the gerbil hippocampus [37]. As AAV2 has been shown to readily transduce striatal neurons, whether the differences in expression levels in the two studies is a consequence of different titers or whether they reflects differences in tropism of the promoter for particular brain areas is not clear.

3. Cellular promoters

As a consequence of the decrease in expression associated with viral-derived promoters, cellular promoters were employed to assess their ability to promote sustained gene expression in the brain. A summary of cellular promoters that have been used with rAAV is presented in Table 2.

3.1. Other non cell-type-specific promoters

The elongation factor 1α (EF1α) gene is a ubiquitously expressed housekeeping gene that plays a pivotal role in protein synthesis and the human EF1α promoter has previously been shown to drive a high level of expression in mouse liver [39]. Lipshutz et al. [40] injected rAAV–EF1α–luciferase intraperitoneally into mice in utero at day 15 of gestation, resulting in sustained expression in the brain for over 6 months. A decrease in luciferase activity after 1 month was attributed to the growth of the pups and an associated dilution of the episomal virus [40]. Xu et al. [41] measured rAAV–EF1α-driven luciferase activity in various brain areas after 2 weeks and found expression comparable to that driven by the glial fibrillary acidic protein (GFAP) promoter, 8–18 times higher than that of the CMV promoter, and 2–8 times lower than that of the NSE promoter.

3.2. Neuron-specific promoters

The rat neuron-specific enolase promoter is probably the most well characterized neuronal promoter that has been used in the context of rAAV-mediated expression in the brain. This promoter was initially shown to drive a high level of neuronally restricted *lacZ* expression [42] in a transgenic mouse study. NSE was found to facilitate a very high level of rAAV-mediated expression in the CNS when utilized by Peel et al. [43] to transduce the rat spinal cord, facilitating GFP expression in thousands of spinal cord neurons. Subsequently, this promoter has also been demonstrated to promote a high level of sustained expression in the striatum [41,45], medial septum [44], substantia nigra [29,33,41,44], and hippocampus [33,41,44]. In a study comparing NSE to the CMV promoter, expression driven by CMV was 8.5-fold lower after 3 weeks and barely detectable at 3 months [44], whereas NSE-driven expression remained stable. Demonstration of the ability of the NSE promoter to drive long-term sustained expression was solidified by Klein et al. [29,33] who observed no attenuation of NSE-driven expression for over 1 year in the basal forebrain and for over 25 months in the substantia nigra.

Xu et al. [41] compared the ability of NSE and eight other promoters to generate luciferase expression 2 weeks postinjection in the rat cortex, hippocampus, substantia nigra, and striatum. NSE was significantly higher in all brain areas, being up to 69 times higher than CMV and up to 8 and 20 times higher than EF1α and GFAP, respectively.

Another neuron-specific promoter that has been used in conjunction with rAAV is the human platelet-derived growth factor B chain (PDGF) promoter which was shown to specifically target gene expression to neurons in transgenic mouse studies [46].

A rAAV cassette with PDGF controlling GFP expression efficiently transduced spinal cord neurons with one GFP-expressing neuron per 45 infectious particles [43]. By comparison, NSE was three times more efficient than PDGF with one GFP-expressing neuron observed

Table 2
Use of cellular promoters for rAAV-mediated transduction of the CNS

Promoter	Transgene	Virions injected	Brain area(s) targeted	Expression level and duration	Reference
EF1α	Luc	3×10^{11} particles	i.p. injection into day 15 mice in utero	Luc assay showed expression stable >6 months in brain although expression in brain lower than that in other organs	[40]
EF1α	Luc	2.5×10^{9} particles	Rat cortex, striatum, substantia nigra, hippocampus	EF1α-Luc activity 8–18 times higher than CMV-Luc and, 2–8 times lower than NSE-Luc.	[41]
NSE	GFP	4×10^{4} IU	Rat cervical spinal cord	857 GFP +ve cells counted (every third 40-μm section counted); stable over 15-week experiment; 1 GFP +ve cell detected per 15 IU of virus	[43]
NSE	GFP	6×10^{5} IU	Rat hippocampus, medial septum, substantia nigra	3×10^{3}–1.5×10^{4} GFP +ve cells in medial septum and substantia nigra and stable over 19 months. 2×10^{3}–3×10^{3} GFP +ve cells in hippocampus at 3 weeks; 8.5-fold higher than CMV–GFP	[44,29]
NSE	Luc	2.5×10^{9} particles	Rat cortex, striatum, nigra, hippocampus	Expression stable over 15-month experiment as measured by luciferase assay. NSE-Luc activity 2- to 8- and 2- to 20-fold higher than those driven by GFAP and EF promoters, respectively.	[41]
NSE	GFP	1.4×10^{10} capsids	Rat striatum	415 GFP +ve cells per 35-μm section transduced over 4-mm area after 3 weeks	[45]
NSE	GFP	5×10^{9} particles	Rat hippocampus and substantia nigra	6×10^{3} GFP-expressing cells detected in hippocampus after 1 month. 2.3×10^{4} GFP +ve cells detected in substantia nigra after 25 months	[33]
PDGF	GFP	4×10^{4} IU	Rat cervical spinal cord	309 GFP +ve cells counted (every third 40-μm section counted); stable over 15-week experiment; 1 GFP +ve cell per 45 IU of virus	[43]
PDGF	GFP-2A-α-synuclein	1×10^{5} IU	Rat substantia nigra	IHC revealed numerous α-synuclein and GFP +ve cells throughout the substantia nigra after 4 weeks (NQ)	[47]
MBP	GFP	6×10^{9} particles	Mouse corpus callosum, striatum, thalamus, cortex	1×10^{3} GFP +ve cells resembling oligodendrocytes in corpus callosum, expression stable over 3-month experiment; <50 GFP +ve cells detected in striatum, thalamus, and cortex	[53]
GFAP	Luc	2.5×10^{9} particles	Rat hippocampus, striatum, cortex, substantia nigra	Luc activity 3–20 times higher than that of CMV-driven luc expression and 2–20 times lower than that of NSE-driven luc expression; 95% of expression appeared neuronal	[41]
GFAP	GFP	4×10^{7} IU	Rat spinal cord	In normal spinal cord most cells transduced were neurons; however, in injured spinal cord, 15–30% of transduced cells were astrocytes	[29]

See note to Table 1 for details and abbreviations. Luc, luciferase.

for every 15 infectious particles injected; however, PDGF was still significantly more efficient at driving gene expression than CMV [43]. Interestingly, PDGF did transduce motorneurons in the cervical enlargement more efficiently than NSE [29]. rAAV–PDGF has also been shown to promote transgene expression throughout the rat substantia nigra for at least 1 month postinjection [47].

Improvements in rAAV expression could emerge from the utilization of multimeric repeats of *cis*-regulatory elements from endogenous promoters. Such an element has not yet been evaluated in an rAAV setting. However, a synthetic promoter containing eight copies of a noradenergic-specific *cis*-regulatory element (PRS2) from the human dopamine β-hydroxylase (hDBH) promoter has been shown to direct >50 higher levels of transgene expression than the hDBH promoter in vitro, and, furthermore, drives high levels of adenovirus-mediated expression specifically in noradrenergic neurons in the rat locus coeruleus [48]. In addition to enhancing gene expression, at 300 bp the small size of this element is ideal for use with rAAV, where size is an important factor in expression cassette design.

3.3. Glia-specific promoters

In a transgenic mouse model, the human glial fibrillary acidic protein promoter was shown to direct expression specifically to astrocytes [49].

In the context of rAAV, however, very few transduced astrocytes were observed by Xu et al. [41] after injection of a reporter gene under control of the GFAP promoter into the hippocampus and less than 5% of striatal cells transduced with the same vectors were morphologically characteristic of astrocytes or colabeled with GFAP. Most GFP positive cells appeared neuronal and colabeled with neuronal marker NeuN. Peel and Klein [29] injected rAAV–GFAP–GFP into the spinal cord and detected expression primarily in neurons; however, in damaged spinal cord, the incidence of astrocytic expression increased to 15–30%. The expression of AAV–GFAP-driven cassettes in neurons was unexpected based on the exclusively astrocytic expression seen in transgenic mouse studies [49]. The AAV inverted terminal repeats contain promoter activity [50,51] which may have contributed to the observed expression.

An oligodendrocyte-specific promoter has also been used to drive rAAV expression. The myelin basic protein (MBP) promoter was originally demonstrated to restrict transgene expression specifically to myelin, forming oligodendrocytes in a transgenic mouse model [52]. Chen et al. [53] injected a rAAV–MBP cassette containing the GFP reporter gene into the mouse corpus callosum, resulting in transduction of oligodendrocytes with expression persisting for greater than 3 months. Injection into the gray matter yielded scarce GFP expression. All GFP transduced cells were colabeled with the oligodendrocyte marker CNPase but not with the neuronal marker NeuN and were morphologically characteristic of type 1 oligodendrocytes [53].

4. The woodchuck posttranscriptional regulatory element

The woodchuck hepatitis virus posttranscriptional regulatory element is a posttranscriptional enhancer that facilitates cytoplasmic accumulation and translation of mRNA [54,55]. In the context of AAV, WPRE was first utilized in vitro to give up to a sixfold improvement in GFP expression. Paterna et al. [56] described the first use of WPRE in vivo by comparing GFP expression from constructs under control of either the PDGF or the CMV promoters with or without the WPRE element. Matched viruses were injected into the substantia nigra and the amount of GFP expression in tyrosine hydroxylase positive neurons was determined. The addition of WPRE to the PDGF–GFP cassette resulted in almost twofold more GFP expression, while addition of WPRE to CMV had no effect. High levels of expression were maintained long term with the WPRE vectors, with expression at 41 weeks similar to that found 4 weeks postinjection [56].

Xu et al. [41] confirmed the expression-enhancing properties of WPRE in other regions of the rat brain, showing that addition of WPRE to NSE-driven cassettes resulted in a 4- to 9-fold increase in luciferase expression in the rat striatum, hippocampus, cortex, and nigra. In agreement, Klein et al. [33] recently demonstrated by Western analysis that addition of WPRE to an AAV–CBA–GFP cassette increased expression 11-fold in the rat hippocampus.

5. Cell-type specific tropism of rAAV

Entry of rAAV into the cell is dependent on binding to the cell surface receptor heparan sulfate proteoglycan (HSPG) [57] and coreceptors fibroblast growth factor receptor-1 (FGFR-1) [58] or αVβ5 [59]. Data gathered from numerous studies have provided overwhelming evidence of the strong tropism of the rAAV2 particle for neurons and this tropism appears, in most cases, to be independent of the promoter used. Most studies reported that approximately 5% or less of transduced cells appeared to be nonneuronal [2–4,16,18,41], and no glial cells were detected when using a neuron-specific promoter [43,44]. This neuronal specificity was demonstrated in an elegant study by Bartlett et al. [60] who distinguished uptake of virus from promoter activity by fluorescently labeling the AAV particle and observed that AAV was preferentially taken up into neurons in the rat brain.

The main exception to this finding in the literature is the oligodendrocyte-specific tropism of the MBP

promoter which was reported to almost exclusively transduce type II oligodendrocytes when injected into the white or gray matter [53]. This is a curious finding based on our knowledge of the strong neuronal tropism of AAV. Bartlett et al. [60] did not observe uptake of AAV particles into astrocytes or oligodendrocytes; however, some astrocytic expression has been observed [11,29], suggesting that glial uptake of AAV can occur under some circumstances. The MBP promoter appeared to be transcriptionally inactive in neurons; therefore, the oligodendrocyte transduction may reflect the 1–5% of glial transduction that other researchers have documented and/or altered tropism in response to needle injury.

Additional insight into transduction of nonneuronal cell types comes from a study by Tenenbaum et al. [11] who injected rAAV–CMV–GFP into the posterior striatum and observed transduction of cells with a glial-like morphology in the internal capsule, an area that lacks neurons. In addition, transduction of glial cultures with rAAV has been well documented [61]. The possibility exists that when there is a sparse neuronal population or when a brain area is enriched in reactive astrocytes, such as after an insult, the absence of neurons allows astrocytes the opportunity to take up the virus.

The HSPG receptor is distributed widely throughout the brain. The FGFR-1 coreceptor is expressed more highly in some brain areas than others and at a higher level in astrocytes than in neurons [62]. Additional coreceptor(s) that have yet to be identified may exist, allowing the possibility that differing levels of coreceptors in particular tissues and cell types may contribute to the ability of specific cell types in different brain areas to take up AAV.

Of the six described serotypes of AAV, only AAV2 gene transfer has been extensively characterized in the brain. Recently it was demonstrated by Davidson et al. [36] that AAV4 and AAV5 are also effective, with expression mediated by these two serotypes being significantly higher than that of rAAV2 in ependymal cells and in the striatum. Much of the AAV5-mediated expression was found in neurons; however, many transduced astrocytes were also evident in the striatum, cortex, and corpus callosum. This altered tropism is not surprising considering that AAV5 binds sialic acid which is not bound by AAV2 [63] and does not bind the AAV2 receptor HSPG [64]. The use of AAV serotypes that have an increased tropism for astrocytes may allow researchers to make more use of glial-specific promoters in driving a high level of expression to treat disease models where transduction of glia is favored.

6. Conclusion

The main limitation of using rAAV is a packaging size limit of around 4.7 kb which prohibits the packaging of expression cassettes over approximately 4.4 kb (excluding the ITRs). To help overcome this obstacle, *trans*-splicing vectors that allow cassettes of twice wild-type AAV size to be packaged have been developed [65–68]. Although these vectors hold great promise for future rAAV studies, the current efficiency has been determined to be only 4–7% of that seen with a single vector containing a full-length gene [69]. Thus, decreasing the promoter size while maintaining promoter strength is essential.

To date there has been only one comprehensive study comparing AAV promoter strengths in the brain [38]. There is inherent difficulty in comparing results from different research groups due to different titering methods, brain areas targeted, routes of administration, and vector purity. To give a rough idea of the relative amounts of vector used in all studies presented here, titers and routes of administration were noted; however, due to the reasons stated above, the level of transgene expression resulting from injection of a particular virus titer cannot be directly compared from one study to the next.

The strongest promoters used with rAAV to date appear to be the NSE and CBA promoters. The NSE promoter has been better characterized in more brain areas and over a longer period of time than the CBA promoter, although it has been found in one study to drive threefold lower expression than CBA [33]. A disadvantage of using NSE is its relatively large size of 1.8–2.2 kb [41,44] which precludes the packaging of large transgenes. When targeting neuronal expression in the brain, the inherent neuronal tropism of rAAV allows advantage to be taken of smaller cell-specific promoters such as CBA. Our laboratory uses a 1.1-kb CBA promoter, allowing an additional 0.7–1.1 kb to be packaged over NSE which improves general utility. Other research groups [31,33] have used a 1.7-kb CBA promoter containing a larger intron which still allows 0.1–0.5 kb to be packaged over NSE. Whether the larger intron in the 1.7-kb CBA promoter provides increased expression levels over the 1.1-kb CBA promoter has not been tested.

The addition of WPRE will increase expression severalfold; however, it also adds an additional 600 bp to the cassette which, allowing for a polyadenylation signal of 300 bp, can accommodate a transgene of up to 1.6–2.0 kb.

Although the level of transgene expression has been greatly enhanced over that of early studies where modest levels of unstable expression directed by the CMV promoter were achieved, researchers remain constantly on the lookout for shorter, stronger promoters to further optimize AAV-mediated expression in the brain.

References

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